JOURNAL OF VIROLOGY, Sept. 2003, p. 9979–9986 Vol. 77, No. 18
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.18.9979–9986.2003
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cis-Acting Elements Required for Efficient Packaging of Brome Mosaic Virus RNA3 in Barley Protoplasts

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Received 24 March 2003/Accepted 27 June 2003

Brome mosaic virus (BMV) is a positive-sense RNA plant virus, the tripartite genomic RNAs of which are separately packaged into virions. RNA3 is copackaged with subgenomic RNA4. In barley protoplasts coinoculated with RNA1 and RNA2, an RNA3 mutant with a 69-nucleotide (nt) deletion in the 3′-proximal region of the 3a open reading frame (ORF) was very poorly packaged compared with other RNA3 mutants and wild-type RNA3, despite their comparable accumulation in the absence of coat protein. Computer analysis of RNA secondary structure predicted two stem-loop (SL) structures (i.e., SL-I and SL-II) in the 69-nt region. Disruption of SL-II, but not of SL-I, significantly reduced RNA3 packaging. A chimeric BMV RNA3 (B3Cmp), with the BMV 3a ORF replacing that of cucumber mosaic virus (CMV), was packaged negligibly, whereas RNA4 was packaged efficiently. Replacement of the 3′-proximal region of the CMV 3a ORF in B3Cmp with the 3′-proximal region of the BMV 3a ORF significantly improved packaging efficiency, and the disruption of SL-II in the substituted BMV 3a ORF region greatly reduced packaging efficiency. These results suggest that the 3′-proximal region of the BMV 3a ORF, especially SL-II predicted between nt 904 and 933, plays an important role in the packaging of BMV RNA3 in vivo. Furthermore, the efficient packaging of RNA4 without RNA3 in B3Cmp-infected cells implies the presence of an element in the 3a ORF of BMV RNA3 that regulates the copackaging of RNA3 and RNA4.

Viral RNAs are specifically selected for packaging during viral infection. Specific packaging occurs through an interaction between viral RNAs and structural coat proteins (CPs). The specific recognition of viral RNAs by CPs plays a crucial role in diverse facets of the viral life cycle and in the packaging event. The binding of CP to specific RNA elements is required for viral protein translation during infection initiation in alfalfa mosaic virus (28) and for the regulation of translation and the initiation of RNA packaging in the RNA phages (38). In retroviruses, nucleocapsid protein is thought to stimulate genomic RNA dimerization, which is required for efficient RNA packaging, reverse transcription, and recombination (14, 31). Many plant viruses require RNA packaging for systemic spread and for cell-to-cell movement (3, 10). Therefore, the CP-RNA interaction is a critical event in the viral life cycle. Compared with the characterization of *trans*-acting factors such as CP in RNA packaging, however, the RNA elements involved in specific packaging have not been well characterized for many viruses. One of the best-characterized RNA elements is "origin of assembly" of tobacco mosaic virus (36, 40). *cis*-acting sequences for RNA packaging have also been demonstrated in several icosahedral viruses, including flock house virus (39), Sindbis virus (37), hepatitis B virus (17), the human immunodeficiency viruses (2, 14, 22), and turnip crinkle virus (32).

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Brome mosaic virus (BMV) is an icosahedral plant RNA virus and is the type member of the genus *Bromovirus* in the family *Bromoviridae* in the alphavirus-like superfamily (18). The genome of BMV consists of three species of messenger sense single-stranded RNA (1). RNA1 (3.2 kb) and RNA2 (2.9 kb), which encode the 1a and 2a replicase proteins, respectively (1, 13, 19), are packaged separately into individual particles (21). RNA3 (2.1 kb), which encodes the 3a cell-to-cell movement protein (35), is packaged into a single particle together with subgenomic RNA4 (0.9 kb) (21). RNA4 is synthesized from the minus strand of RNA3 (25) and encodes CP. CP is required for packaging, cell-to-cell movement, and the systemic spread of the virus (29, 33, 34).

A highly conserved N-terminal arginine-rich motif in BMV CP plays an important role in BMV RNA packaging through RNA-CP interactions (4, 5, 33, 34). The crystallographic structure of BMV virions has been determined (23). RNA regions or elements involved in the packaging of BMV RNAs have been assigned to the coding region of BMV RNA1 by UV cross-linking and band-shift assays (11) as well as to the 3′-proximal region of the 3a open reading frame (ORF) in RNA3 (9). The tRNA-like structures (TLS) in the 3′-untranslated regions of BMV RNAs also play a crucial role in BMV RNA packaging in vitro (6). In the present study, we delimit a nucleotide sequence required for the efficient packaging of BMV RNA3 and show that 69 nucleotides (nt) in the 3′-proximal region of the BMV 3a ORF, especially a predicted stem-loop structure (30 nt), is essential for the efficient packaging of BMV RNA3. We also propose the presence of elements in the BMV 3a ORF that are involved in the regulation of the copackaging of RNA3 and RNA4.